

Characterization of the *in vivo* and *in vitro* Effects of Indomethacin on Human Natural Killer Cell Activity

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The *in vivo* and *in vitro* effects of indomethacin on the natural killer (NK) cell activity against K 562 target cells were studied. *In vivo* administration of indomethacin, 3×50 mg for 7 days to normal donors did not influence baseline NK cell activity, which means that treatment with prostaglandin (PG) inhibitors can be allowed in studies on NK cell activity of persons with normal PG production. The NK cell activity of fresh mononuclear cells was boosted with pharmacological concentrations of indomethacin *in vitro*, while frozen cells were not. Our results indicate that indomethacin enhances the NK cell activity *in vitro* by blocking the prostaglandin production of monocytes, since monocyte depleted effector cells were not boosted by indomethacin.

Key words: natural killer cell activity; frozen cells; indomethacin; prostaglandins.

Accepted for publication 2 June 1986

A number of agents are known to influence the level of NK cell activity (9, 11). Interferon (IF) (2, 10) and interleukin 2 (IL-2) (8, 15) augment the cytolytic function of NK cells, while agents shown to depress NK cell activity include cAMP (6), phorbol esters (6), azathioprine (13), glucocorticosteroids (14) and prostaglandins (PG) (1, 3, 4). Addition of PGE₁, PGE₂, PGA₁ or PGA₂ to cytotoxic assays results in marked depression of NK cell activity, whereas addition of PGB₁, PGB₂, PGF₁ or PGF₂ induced little or no inhibition (1). *In vitro* it has been shown that drugs which inhibit PG production, e.g. indomethacin and aspirin, enhance both natural and antibody dependent cell cytotoxicity (3). One way of enhancing NK cell activity may therefore be through an inhibition of PG production. The *in vivo* effect of PG inhibitors on human NK cell function is, however, not known. In the present communication we describe the *in vivo* and *in vitro* effects of indomethacin on the cytolytic function of human NK cells from normal donors.

MATERIAL AND METHODS

In vivo and in vitro effects of indomethacin on NK cell activity in normal persons

Eight healthy males were given indomethacin 3×50 mg/day for 7 days. Baseline NK cell activity and *in vitro* boosting of the NK cells with indomethacin (1 µg/ml) were made before, on day 7 of indomethacin treatment (1 h after intake of last indomethacin dose) and 7 days after indomethacin treatment was stopped. Baseline NK cell activity and NK cell activity after *in vitro* boosting with indomethacin were studied in parallel in eight healthy male controls not receiving any medication, in order to evaluate the day-to-day variation. Mean age of the indomethacin group was 34.8 years (range 32-46), while that of the controls was 31.1 (range 23-35).

Determination of NK cell activity

Effector cells. All blood samples were collected

between 7 and 9 a.m. Heparine (125 IU/ml) was used as anticoagulant. Mononuclear cells were isolated immediately after blood sampling by Ficoll Isopaque gradient centrifugation. The cells were washed three times with Hanks' buffered salt solution and resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) to final concentrations of 4×10^6 , 2×10^6 and 10^6 cells/ml. In some experiments 1.5 ml of 4×10^6 cells/ml were incubated at 37°C on 60 mm Petri dishes (Teknunc A/S, Roskilde, Denmark) for 1 h. The nonadherent cells were aspirated, centrifuged (1000 g , 10 min) and resuspended in RPMI with 10% FCS. A 10 ml plastic syringe was filled with 2 g nylon wool (Fenwall laboratories). The column was washed twice with 15 ml RPMI with 10% FCS. After preincubation at 37°C , the column was ready for use. 2 ml of 4×10^6 cells/ml were applied on the nylon column and overlaid with 1 ml medium. After 30 min of incubation at 37°C , the cells were passively eluted by 20 ml 37°C RPMI with 10% FCS. The preparation contained less than 1% peroxidase positive cells.

Mononuclear cells from some of the donors were frozen ($1^\circ\text{C}/\text{min}$, controlled gradient freezing machine Cryoson, GB) in RPMI with 20% FCS and 10% dimethylsulphoxide and stored in liquid nitrogen until use. To avoid day-to-day variation, cells from a given donor isolated before, during and after indomethacin treatment and cells from the healthy control were thawed on the same day and tested in parallel.

Target cells. The K 562 cell line (an erythromyeloid cell line derived from pleural effusion of a patient with chronic myelocytic leukemia in blast crisis) was maintained in continuous suspension culture in RPMI 1640 supplemented with 10% heat-inactivated FCS. To $2-8 \times 10^6$ target cells in 1 ml were added $0.1 \mu\text{Ci}$ of $\text{Na}_2^{51}\text{CrO}_4$ (New England Nuclear, Boston Mass, U.S.A.) specific activity 350–600 mCi/mg. The cells were incubated with the isotope for 1 h at 37°C . The labelled cells were washed through a 4 ml cushion of heat-inactivated FCS. The cell pellet was resuspended in RPMI with 10%

heat-inactivated FCS to a final concentration of 5×10^4 cells/ml.

Cytotoxic assay. NK cell activity was measured at three effector/target (E/T) cell ratios (80/1, 40/1, 20/1). Some experiments were carried out only at E/T = 80/1. 400 μl of the labelled target cell suspension was mixed with 400 μl of effector cells and 200 μl of RPMI + 10% heat-inactivated FCS. After incubation for 4 h at 37°C the tubes were centrifuged (1000 g , 10 min), 500 μl of each supernatant was transferred to new tubes, and the radioactivity was counted in a Packard scintillation gamma counter. The experimental release was calculated by the formula:

$$\frac{{}^{51}\text{Cr activity released in the supernatant}}{{}^{51}\text{Cr total activity}} \times 100\%$$

The specific lysis (NK cell activity) is the difference between experimental release in the presence of effector cells and experimental release when target cells were incubated with medium only.

Incubation of effector cells with indomethacin

Indomethacin (Confortid, DUMEX) was dissolved and diluted in RPMI. 4×10^6 cells/ml were preincubated for 1 h at 37°C with indomethacin which was also present during the incubation with target cells. The final concentration of indomethacin was $1 \mu\text{g}/\text{ml}$ in most experiments. In one series of experiments effector cells were incubated with indomethacin at various concentrations. In another series of experiments different incubation times of indomethacin were investigated. Possible toxic effects of indomethacin were tested by staining the effector cells with Nigrosin 0.02% (w/v). After 5 h of incubation with the drugs cell viability was above 95%.

Statistical evaluations

The percentage of ^{51}Cr release was calculated for samples made in triplicate (SD < 3.0%).

Table 1

In vivo and *in vitro* effects of indomethacin on NK cell activity in normal persons (means $\pm$ SD)

Treatment period	Effector cells	<i>In vitro</i> indomethacin	Before	During	After
				NK activity % specific lysis	
Indomethacin group (<i>n</i> = 8)	fresh	-	25.3 $\pm$ 10.4	22.1 $\pm$ 8.4	27.4 $\pm$ 17.1
	fresh	+	35.4 $\pm$ 13.0	38.2 $\pm$ 12.3	37.9 $\pm$ 17.8
	frozen	-	28.2 $\pm$ 14.8	24.9 $\pm$ 11.4	23.9 $\pm$ 9.0
	frozen	+	28.9 $\pm$ 14.5	25.0 $\pm$ 12.9	27.0 $\pm$ 12.3
	fresh	-	19.0 $\pm$ 9.6	16.5 $\pm$ 9.1	18.6 $\pm$ 9.7
Control group (<i>n</i> = 8)	fresh	+	29.4 $\pm$ 11.5	27.1 $\pm$ 11.3	28.7 $\pm$ 13.4
	frozen	-	16.9 $\pm$ 9.7	15.8 $\pm$ 8.2	16.7 $\pm$ 9.2
	frozen	+	20.0 $\pm$ 11.0	19.1 $\pm$ 9.6	21.6 $\pm$ 10.4

The significance of the observed differences was evaluated by the Mann-Whitney and Wilcoxon tests.

RESULTS

In vivo and *in vitro* effects of indomethacin on NK cell activity in normal persons

There was no statistically significant difference in baseline NK cell activity or in the *in vitro* indomethacin boosting of fresh NK cells between the group receiving indomethacin treatment and the control group (Table 1). Baseline NK cell activity of fresh and frozen cells was not significantly different. The effect of *in vitro* indomethacin boosting on NK cell activity of frozen cells from both test persons and controls was significantly less than that obtained with fresh cells ($P < 0.01$). PGE1 and PGE2 at 10^{-4} to 10^{-6} M totally inhibited NK cell activity of both frozen and fresh cells, while PGE1 and PGE2 in the concentration 10^{-7} M only inhibited the NK cell activity of fresh cells, without any effect on frozen cells (data not shown).

Further characterization of the in vitro effect of indomethacin on NK cell activity in normal persons

Monocyte-depleted effector cells were boosted by indomethacin to a significantly lesser extent than were mononuclear cells ($P < 0.01$) (Table 2).

The enhancing effect of different concentrations of indomethacin on NK cell activity is shown in Table 3. When a group of experiments was assessed for the effect of indomethacin on the enhancement of cytotoxicity, the results were significantly different from con-

Table 2

In vitro effect of indomethacin on NK cell activity of monocyte-depleted effector cells

	NK activity % specific lysis	
	Mononuclear cells	Monocyte depleted cells
- Indomethacin	24.7 $\pm$ 7.9	26.5 $\pm$ 11.7
+ Indomethacin	32.1 $\pm$ 8.6	28.1 $\pm$ 12.2
Augmentation (%)	30.5	6

Means $\pm$ SD of seven experiments are shown.

Table 3

Effect of different concentrations of indomethacin on the NK cell activity

Indomethacin μ g/ml	0	0.001	0.005	0.01	0.05	0.1	0.5	1	5	10
NK activity %	100	97	104	109	116	132	157	149	158	151

Mononuclear cells were preincubated with various concentrations of indomethacin before testing for NK cell activity. Results are given in percentage of control - one representative experiment of five is shown.

trols at the $P < 0.01$ level for indomethacin 1 $\mu\text{g/ml}$ (30 experiments), $P < 0.01$ for indomethacin 0.1 $\mu\text{g/ml}$ (10 experiments) and $P < 0.05$ for indomethacin 0.01 $\mu\text{g/ml}$ (8 experiments).

The results of time course studies showed that enhancement of cytotoxicity by indomethacin required 2½ h of incubation or more for significant enhancement to be seen. If the period of incubation with indomethacin was extended cytotoxicity increased (Table 4). Pre-incubation of effector cells with indomethacin before incubation with target cells further augmented cytotoxicity.

The effect of indomethacin on NK cell activity was reversible, as washing of the effector cells after incubation with indomethacin abolished the enhancing effect (data not shown).

DISCUSSION

Our results showed that indomethacin administered for 7 days to normal persons did not influence baseline NK cell activity. NK cell activity could be boosted with indomethacin *in vitro* to a similar degree in persons receiving the drug and in controls.

To eliminate day-to-day variation only male donors were examined, as it was recently found that NK cell activity rises during the menstrual cycle (16). To avoid day-to-day variation due to fluctuations in the NK sensitivity of the target cell line, frozen cells collected before, during and after indomethacin treatment were thawed on the same day and tested for NK cell activity. Furthermore, controls not receiving any medication were tested in parallel.

Brunda et al. (1) showed that when tumor-bearing mice were given indomethacin in their drinking water, there was a partial or complete restoration of NK cell activity. Likewise a single injection of aspirin completely or partially restored NK cell activity to levels found in normal mice. Injection of aspirin into normal mice had little or no effect on NK cell activity. Our results show that indomethacin treatment of normal human individuals does not influence NK cell function. It is, however, possible that indomethacin increases the NK cell activity of persons with high PG production, e.g. cancer patients or patients with chronic immune inflammatory diseases. We have previously examined the NK cell activity of 45 patients with rheumatoid arthritis, not receiving any remission inducing therapy (12). These patients were treated with various PG inhibitors, which did not seem to influence NK cell activity, but longitudinal studies on the NK cell activity before, during and after treatment with PG inhibitors have not been performed.

The effect of indomethacin on the function of NK cells was shown to be reversible, since washing of the effector cells abolished the enhancing effect of indomethacin. We therefore can not exclude the possibility that indomethacin *in vivo* actually increases NK cell activity, but that we are unable to measure this possible enhancement, as the indomethacin bound *in vivo* to the cells might be washed away during the isolation of the mononuclear cells.

The *in vitro* experiments were performed using pharmacological concentrations of indomethacin (1 $\mu\text{g/ml}$) (5), but it was shown that even sub-pharmacological concentrations of indomethacin (0.1–0.01 $\mu\text{g/ml}$) also enhance NK cell activity.

Table 4
Time course of the effect of indomethacin on NK cell activity

Hours	0	½	1	1½	2	2½	3	3½	4	*1 + 4
NK activity	100	102 ± 6	108 ± 9	125 ± 11	123 ± 13	137 ± 7	151 ± 10	151 ± 12	161 ± 18	168 ± 11
Significance		NS	NS	NS	NS	$P < 0.05$		$P < 0.01$		

Assay tubes contained effector cells and target cells (ratio 80/1) plus indomethacin (1 $\mu\text{g/ml}$). As indicated results are given in percentages of controls (mean $\pm$ SD of six experiments). * 1 h of preincubation + 4 h of incubation with target cells.

Our *in vitro* studies indicate that indomethacin enhances NK cytotoxicity through an inhibition of the PG production by monocytes, as monocyte-depleted effector cells were not boosted by indomethacin.

The finding that NK cell activity of frozen mononuclear cells was not boosted by indomethacin and that PGE₁ and PGE₂ 10⁻⁷ M did not inhibit frozen cells indicates that the PG sensitivity of NK cells that have been frozen and thawed is decreased. In addition, the PG production by the monocytes might be disturbed by the freezing procedures. In future studies on the influence of PG's on NK cell function, one should not use frozen cells.

A clinically important conclusion from our findings is that treatment with PG inhibitors can be allowed in studies on NK cell activity of persons with normal PG production. It would be of great interest in future studies to examine whether PG inhibitors are able to modify immune responses in patients with disturbed PG production.

ACKNOWLEDGEMENTS

We thank Dr. V. Andersen for valuable discussions. The excellent technical assistance of Kirsten Ankersø is gratefully acknowledged. The work was supported by the Danish National Association against Rheumatic Diseases.

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